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SPECTROPHOTOMETRIC METHODS FOR THE DETERMINATION OF FLUNIXIN MEGLUMINE AND MENBUTONE IN BULK AND DOSAGE FORMS

FOUAD M.M.[1]*, ABD EL-RAZEQ S.A.[1], BELAL F.F.[2] AND FOUAD F.A.[1]

[1]Analytical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.
[2]Analytical Chemistry Department, Faculty of Pharmacy, Al-Mansoura University, Egypt.
*Corresponding Author: Email- manalfoad2000@yahoo.com

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Abstract- Four simple, accurate and reproducible spectrophotometric methods are presented for the determination of two veterinary drugs, flunixin meglumine (flunixin-M) and menbutone in pure form as well as in pharmaceutical dosage forms. The first one (Method A) depends on the formation of a charge-transfer complexes between flunixin-M as n-donor and 2,3-dicholro-5,6-dicyano-1,4-benzoquinone (DDQ), chloranilic acid (CA) and p-Chloranil (CR) as p-acceptors giving colored products quantified spectrophotometrically at 588, 521 and 345 nm, respectively. Beer's plot shows good correlations in the concentration range of 10-100, 50-300 and 10-80 µg mL^{-1}, respectively. The second method (Method B) depends on the nucleophilic substitution reaction between the cited drug and 1,2-naphthoquinone-4-sulfonate (NQS) producing an orange-red colored product measured at 484 nm with a linearity range of 20-120 µg mL^{-1}. The third method (Method C) is based on the formation of ion pair complexes between menbutone and basic fuchsin (BF), methylene blue (MB) and safranin O (SFNO) in buffered aqueous solution at pH 7.5 ±0.5 with the three dyes to give highly colored complex species extractable with chloroform, measured at 557, 650 and 516 nm, respectively. Beer's law is obeyed in the concentration range of 8-40 µg mL^{-1} with BF and SFNO or 1-8 µg mL^{-1} with MB. The fourth method (Method D) is based on the reaction of carboxylic acid group of menbutone with a mixture of potassium iodate and potassium iodide in aqueous medium at room temperature producing yellow free I_2 having a maximum at 350 nm with a linearity range of 3-20 µg mL^{-1}. The suggested methods are used to determine the cited drugs in pharmaceutical formulation; flunidyne or menbutone injections with mean recoveries ranging from 98.98-100.99%. The validity of the methods is further assessed by applying the standard addition technique. The obtained results are statistically analyzed and found to be in accordance with those given by manufacturer methods.

Keywords- flunixin meglumine, menbutone, charge-transfer, naphthoquinone-4-sulfonate, ion pair, KIO_3/KI

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Introduction

Flunixin; 2-{{2-methyl-3-(trifluoro-methyl) phenyl} amino}-3-pyridine carboxylic acid [1], is usually found as its meglumine salt. Its actions are related to its ability to inhibit cyclooxygenase, hence acting as analgesic, antipyretic and anti-inflammatory [2]. Various methods have been reported for its determination including electrochemical [3], GC [4-6], TLC [7] and HPLC [7-10] methods. No spectrophotometric methods have been developed for its determination.

Menbutone; 4-Methoxy-γ-oxo-1-naphthalene butanoic acid [1] is a specific stimulant of exocrine glands of digestive tract; digestive tonic and choloretic. It helps in any course affecting the digestive system as diarrheas, anorexia and gastro-enteritis [11]. Reviewing the Literature, it was found that no analytical methods have been published for the estimation of menbutone.

The aim of the present study is to develop simple and rapid spectrophotometric methods for analysis of flunixin-M or menbutone in pure form and in pharmaceutical formulations.

Materials and Methods

Instrumentation

Analysis was carried on Shimadzu UV/Vis spectrophotometer PC-160, (Tokyo, Japan) and using a Jenco pH meter with Jenway double junction glass electrode (UK).

Pure Samples

Pure flunixin-M, was kindly supplied by Delta Pharma, Egypt. Its purity was found to be 99.9% as stated by the supplier. Pure menbutone was kindly supplied by Egyptian Co. for chemicals and pharmaceutical, Egypt. Its purity was found to be 100.621% according to manufacturer method [12].

Market Samples

Flunidyne injections, B.N. 0846/11, each 1 mL contains 83 mg flunixin-M equivalent to 50 mg flunixin, a product of Arab company for medical products, Egypt. Menbutone 10% injections, B.N. 0.081133, each 1 mL contain 100 mg menbutone, manufactured by

Egyptian Co. for chemicals and pharmaceutical, Egypt, both are purchased from local market.

Chemicals and Reagents

All chemicals and reagents used were of analytical grade, solvents were of spectroscopic grade and freshly distilled water was used throughout the work.

2,3-dicholro-5,6-dicyano-1,4-benzoquinone (DDQ), chloranilic acid (CA) and p-Chloranil (CR) (Merck, Germany); 0.2% solutions in acetonitrile were used. NQS (Merck, Germany); 0.1% solution in distilled water. Basic fuchsin (BF) (Riedell-detlean, Germany); 0.2% aqueous solution, Methylene blue (MB) (Rankem, New Delhi) and Phenosafranin O (SFNO) (Riedell-detlean, Germany); 0.1% aqueous solutions. Phosphate buffer solution (pH 6-10), prepared by mixing 50 mL of 0.2 M KH_2PO_4 with different volumes of 0.2 M NaOH and diluting to 200 mL with distilled water [13].

Standard Solutions

Standard solution of flunixin-M (1.0 mg mL^{-1}) was prepared in acetonitrile for CA. Whereas (0.4 mg mL^{-1}) was prepared by appropriate dilution of the above solution with acetonitrile to be used for DDQ and CR. 0.4 mg mL^{-1} aqueous solution was prepared to be used with NQS.

Standard solution of menbutone (0.4 mg mL^{-1}) was prepared in methanol for BF and SFNO. Whereas 0.1 and 0.05 mg mL^{-1} solutions were prepared by appropriate dilution of the latter solution with methanol to be used for MB and KIO_3/KI method, respectively.

These solutions were stable for about one week if stored in the refrigerator.

General Procedures

Method (A)

For DDQ and CA Methods: Aliquots of standard solution of flunixin -M in acetonitrile (0.4 or 1.0 mg mL^{-1}) containing 0.1-1.0 mg or 0.5-3.0 mg of the drug were transferred into two separate sets of 10-mL volumetric flasks. 2ml of 0.2% DDQ were added to each flask of the first set and 2.5 mL of 0.2% CA were added to the second one then completed to volume with acetonitrile. The absorbance of the red and purple colored products was measured immediately at 588 and 521 nm, respectively against a reagent blank.

For CR: Aliquots of working flunixin-M solution (0.4 mg mL^{-1}) in acetonitrile equivalent to 0.1-0.8 mg were transferred into a set of 20-mL test tubes. 2 mL of 0.2% CR in acetonitile were added to each tube, volumes were completed to 8 mL with acetonitrile and heated in thermostatic water both at 60°C for 10 min. After cooling, the content of each tube was transferred quantitatively to a set of 10 mL volumetric flasks and completed to volume with acetonitrile. The absorbance of the yellow colored product was measured at 345 nm against a reagent blank.

Method (B)

Different volumes of aqueous flunixin-M solution (0.4 mg mL^{-1}) equivalent to 0.2-1.2 mg were accurately transferred into a series of test tubes. 2 mL of 0.1% NQS in water was added to each tube followed by 2.5 mL of 0.1 M $NaHCO_3$. Volume was completed to 8 mL with water, tubes were then placed in a thermostatic water bath at 65°C for 10 min, cooled then transferred quantitatively into 10 mL volumetric flasks and completed with distilled water. The absorbance of the produced orange red color was measured at 484 nm against a reagent blank.

Method (C)

To a set of separating funnels, aliquots equivalent to 0.025-0.2 mg from the standard menbutone solution (0.1 mg mL^{-1}) were transferred. To another two sets volumes equivalent to 0.2-1.0 mg from methanolic drug solution (0.4 mg mL^{-1}) were also transferred. 1.5 mL phosphate buffer of pH 7.5±0.5 were added followed by 3 mL of 0.1% MB to the first set or 0.2% BF, 0.1% SFNO to the second or third sets, respectively. The volume of the aqueous phase was adjusted to 10 mL with distilled water. Two successive extractions were made using 10 mL chloroform for each by shaking for about one minute. The extracts were collected into 25-mL volumetric flasks after passing through anhydrous sodium sulphate and the flasks were set aside for 15 min. Volumes were then adjusted with chloroform. The absorbance of the developed colors was measured at 650, 557 or 516 nm for the three dyes, respectively against a reagent blank.

Method (D)

Into a series of 10 mL volumetric flasks, aliquots of methanolic menbutone solution (0.05 mg mL^{-1}) equivalent to 0.03-0.2 mg drug were introduced. 2 mL of 0.15 M aqueous KI solution were added followed by 2.5 mL of 0.1 M KIO_3 aqueous solution and set aside for 15 min. Solutions were adjusted to mark with distilled water and the absorbance of the yellow color was measured at 350 nm against a drug blank.

Application to Pharmaceutical Formulations

Methods (A) and (B)

Three flunidyne injections were mixed and aliquot of the mixed solution equivalent to 100 mg was transferred to a 100 mL volumetric flask and completed to volume with acetonitrile to obtain a solution claimed to contain 1.0 mg mL^{-1} flunixin-M used for CA method. 10-mL of the above solution was diluted to 25 mL with acetonitrile to obtain a drug solution claimed to contain 0.4 mg mL^{-1} used for DDQ and CR methods. The procedure detailed above was repeated using distilled water as diluent instead of acetonitrile to obtain an aqueous solution claimed to contain 0.4 mg mL^{-1} of the drug to be used for NQS method. Solutions were analyzed following the details under "General Procedures".

Methods (C) and (D)

Three menbutone 10% injections were mixed well. Aliquot of the mixed solution equivalent to 40 mg menbutone was transferred into 100-mL volumetric flask. Volume was completed with methanol to obtain a solution claimed to contain 0.4 mg mL^{-1} menbutone used for BF and SFNO methods. Further dilution of the above solution was done with methanol to obtain a solution labeled to contain 0.1 mg mL^{-1} menbutone for MB method or 0.05 mg mL^{-1} drug solution for KIO_3/KI method. The prepared solutions were then analyzed as detailed under "General Procedures".

Results and Discussion

Method (A)

Flunixin-M was determined spectrophotometrically through charge transfer complexation in which DDQ, CA and CR as p-acceptors reacted with the cited drug to give a charge transfer complexes which dissociate into intensely colored radical anions in the polar solvent [14-17]. The maximum absorbance was measured at 588, 521 or 345 nm, respectively; as shown in [Fig-1] and [Fig-2].

The reaction may be presented by the following equation:

$$D + A \rightarrow [D \rightarrow A] \rightarrow D^+ + A^-$$

D = Donor, A = Acceptor Donor, [D→A] = Donor Acceptor Complex, $D^+ + A^-$ = Radical ions

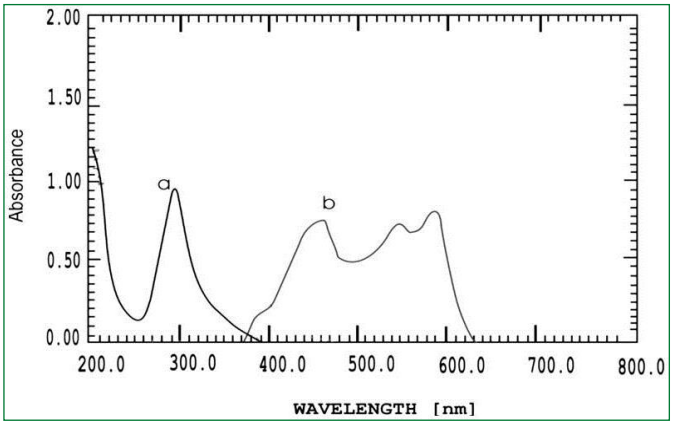


Fig. 1- Absorption spectra of: a. Intact flunixin-M (20 µg mL^{-1}) in acetonitrile, b. Flunixin-M (80 µg mL^{-1})-DDQ reaction product in acetonitrile.

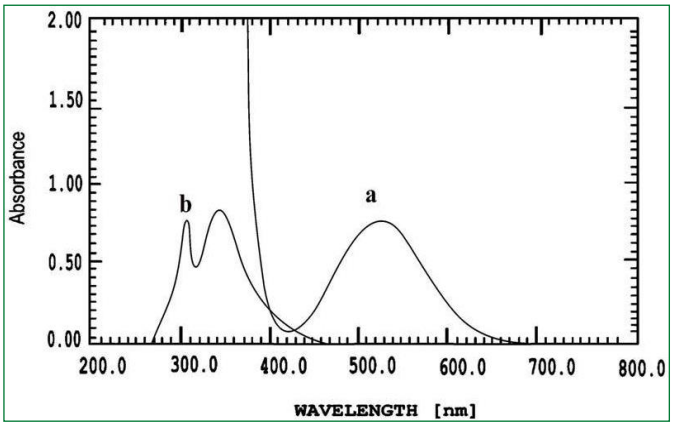


Fig. 2- Absorption spectra of: a. Flunixin-M (250 µg mL^{-1})-CA reaction product in acetonitrile, b. Flunixin-M (60 µg mL^{-1})-CR reaction product in acetonitrile.

Method (B)

Nucleophilic substitution reaction between aqueous solutions of flunixin-M and NQS took place in alkaline medium to give an orange red colored product absorbing maximally at 484 nm [Fig-3].

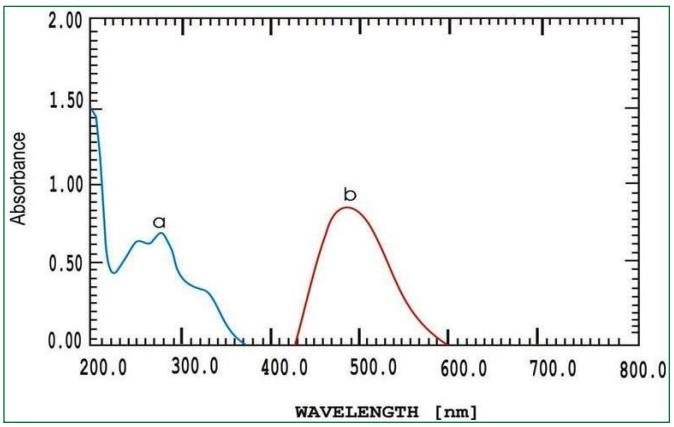
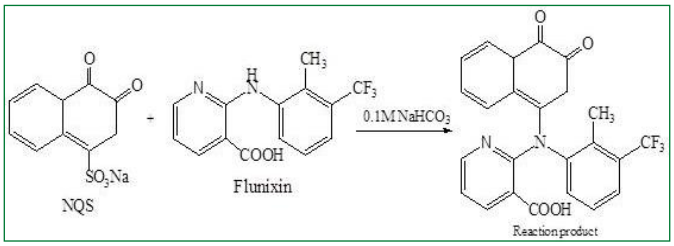


Fig. 3- Absorption spectra of: a. Intact flunixin-M (20 µg mL^{-1}) in water, b. Flunixin-M (100 µg mL^{-1})-NQS reaction product in water.

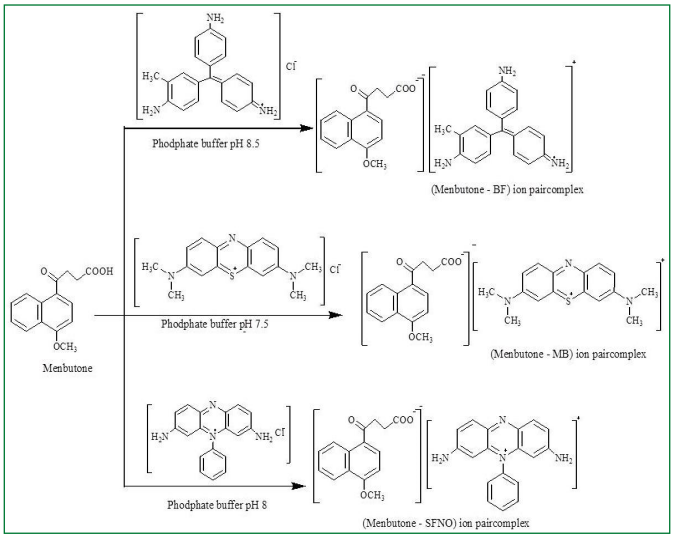
The suggested mechanism can be presented in the following scheme.



Scheme 1- Reaction between flunixin and NQS

Method (C)

Menbutone reacts with BF, MB and SFNO at pH 7.5±0.5 to form highly colored ion-association complexes extracted with Chloroform. The reaction yielded a red, blue or pink colored complex with BF, MB or SFNO with an absorption maximum at 557, 650 or 516 nm, respectively, [Fig-4] and [Fig-5]. The structure of the ion pair association complex is shown in [Scheme-2].



Scheme 2- Ion-association complexes of menbutone with BF, MB and SFNO.

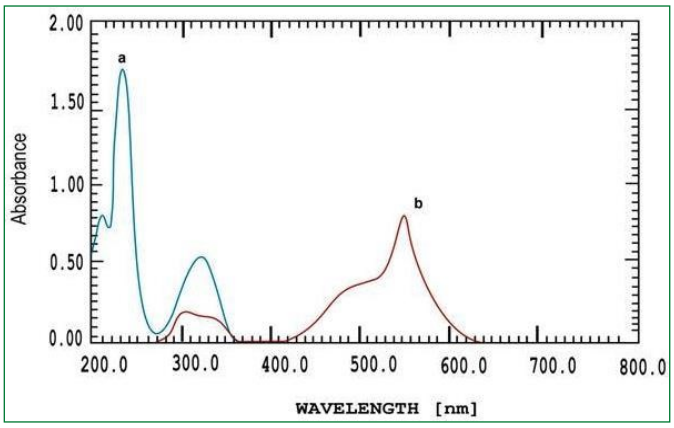


Fig. 4- Absorption spectra of: a. Intact menbutone (8 µg mL^{-1}) in methanol, b. Menbutone -BF ion pair (32 µg mL^{-1}) in chloroform.

Method (D)

It has been reported that iodine is formed as a result of the interaction of a mixture of iodide and iodate with inorganic or organic acid in accordance with the equation: $5I^- + IO_3^- + 6H^+ \rightarrow 3H_2O + 3I_2$

In aqueous medium, the iodide ions react with the liberated iodine to yield triodide ion ($I_2 + I^- \rightarrow I_3^-$) which is detected at 298 and 350 nm [18].

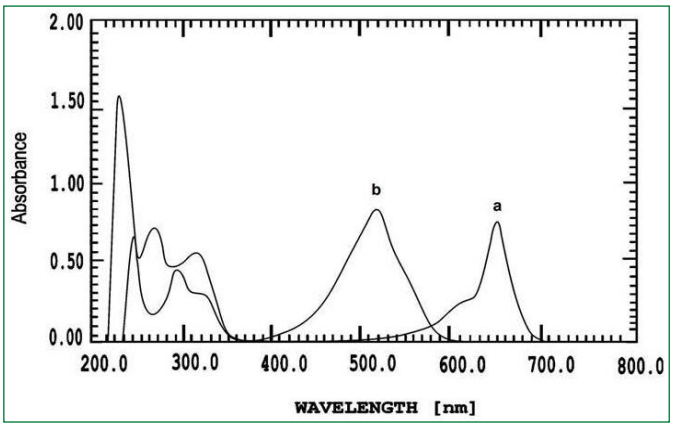


Fig. 5- Absorption spectra of: a. Menbutone -MB ion pair (4 µg mL^{-1}) in chloroform, b. Menbutone-SFNO ion pair (32 µg mL^{-1}) in chloroform.

Accordingly, a reaction of carboxylic acid group of menbutone with a mixture of potassium iodate and potassium iodide in aqueous medium at room temperature took place, producing yellow free I_2 exhibiting an absorption maxima at 288 and 350 nm. The new band at 350 nm was chosen for the quantitative analysis of the drug; [Fig-6]. A suggested reaction pathway was deduced and presented in [Scheme-3].

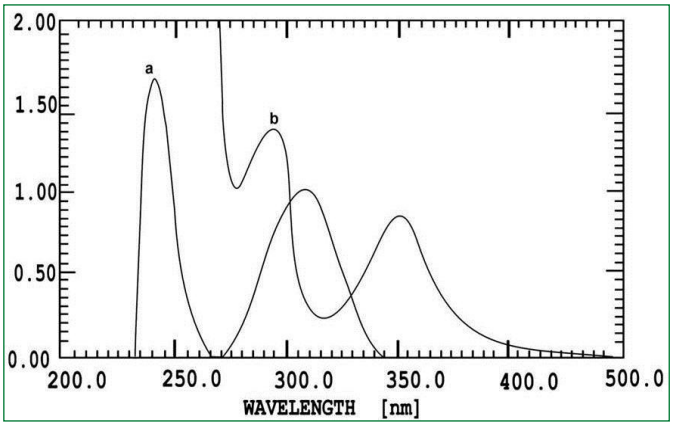
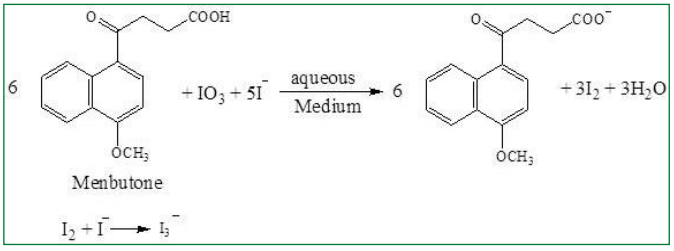


Fig. 6- Absorption spectra of: a. Drug blank (15 µg/mL^{-1}) in water, b. Menbutone (15 µg mL^{-1}) KIO3/KI reaction product in water.



Scheme 3- Suggested reaction of menbutone with KIO_3/KI mixture.

Optimization of the Reaction Conditions

Effect of pH and Buffer Volume

Investigation of the effect of the pH revealed that phosphate buffer of pH 7.5±0.5 was found to be optimal for the ion-pair formation and was used throughout the work with the three dyes.

Effect of Alkalinity

To generate the nucleophiles from flunixin-M and also to activate the nucleophilic substitution reactions between the drug and NQS, alkaline medium was necessary. Different inorganic bases were tested; 0.1M $NaHCO_3$, 10% sodium acetate and 0.1M K_2HPO_4.2.5 mL of 0.1M $NaHCO_3$ was sufficient to give maximum color intensity so was used.

Effect of Reagents Volume

Studying the effect of the volume of reagents used for flunixin-M revealed that 2mL of 0.2% DDQ or 0.2% CA and 2.5 ml of 0.2% CR or 0.1% NQS solutions were found to be optimal for the reaction with the cited drug.

While for menbutone, different volumes of the three dyes were tried, it was found that 3 mL of 0.2% BF, 0.1% MB or 0.1% SFNO were sufficient to produce maximum color intensity. 2.5 mL or 3 mL of 0.1M KIO_3 or 0.15M KI solutions respectively were Optimum for the reaction.

Effect of Time and Temperature

Complete color development was attained instantaneously at room temperature between flunixin-M and DDQ or CA and remained stable for further one hour. However, with CR or NQS the reaction was slow at room temperature, suggesting heating at different temperatures with the two reagents. The highest color intensity was attained after heating at 60°C or 65°C for 10 min and remained stable for further 50 min with the two reagents, respectively.

For menbutone, maximum color intensity was obtained after 15 min with BF and SFNO or after 10 min with MB. Thus 15 min standing time at room temperature was recommended for ion-pair formation. Whereas for the reaction of the drug with KIO_3/KI, upon heating the reaction mixture, it showed a decreased absorbance at the relevant λ_{max}. The mixture was kept at room temperature for different time intervals where maximum color was obtained after 15 min.

Stoichiomery of the Reaction

The stoichiometric ratio of flunixin-M to reagents was determined by molar ratio method [19] using 5x10^{-3} M of the drug with DDQ or CA and 2x10^{-3}M with CR or NQS, where a ratio of 1:1 (drug: reagent) was observed. For menbutone, application of molar ratio [19] of 1:1 (drug: reagent) for the three dyes, and a ratio of 6:1(drug: KIO_3).

Method Validation

The proposed methods were validated in accordance with ICH guidelines [20].

Linearity

Linear relationship was found to exist between absorbance and the corresponding concentration of flunixin-M in the range of 10-100, 50 -300, 10-80 and 20-120 µg mL^{-1} of the drug using DDQ, CA, CR and NQS, respectively. The correction coefficients ranged from 0.9990-0.9999 indicating good linearities [Table-1].

Beer's plots were found to be linear with good correlation coefficients (0.9994-0.9999) in concentration range of 8-40, 1-8, 8-40 or 3-20 µg mL^{-1} menbutone with BF, MB, SFNO or KIO_3 /KI, respectively [Table-2].

Accuracy and Precision

Accuracy and precision of the proposed methods were validated by analyzing four flunixin-M or menbutone concentration levels in triplicates on the same day or on three different days. Accuracies calcu-

lated as R% ± SD were 100.74-99.28 ± 1.92-0.95 or 100.89-99.96±1.37-0.41 with the two drugs, respectively, while precision was calculated as inter and intraday RSD% found to be in the range of 0.36-1.95 or 0.12-1.97 and 0.26-1.56 or 0.25-1.97, respectively; [Table-1] and [Table-2].

Selectivity

The proposed methods were successfully applied for the analysis of flunixin-M or menbutone in flunidyne or menbutone injections with mean recoveries ranging from 99.14-100.99% or 98.98-100.42%; respectively.

Results obtained for the determination of the two drugs were statistically compared to the those obtained by manufacturer UV spectrophotometric methods [12,21], revealing no significant difference; [Table-3] and [Table-4]. The validity of the proposed methods are further assessed by applying the standard addition technique, good recoveries of added were obtained in the range of 96.22-104-12% or 97.90-102.76% for the two drugs respectively; [Table-3] and [Table-4].

Table 1- Assay parameters of the proposed spectrophotometric methods for the determination of flunixin-M.

Parameter	DDQ Method	CA Method	CR Method	NQS Method
λ_{max} (nm)	588	521	345	484
Linearity range (µg mL^{-1})	10-100	50-300	Oct-80	20-120
A (1%, 1cm)	116	30	134	82
Regression parameters Slope ± SD (S_b)	0.0111± 5.8×10^{-5}	0.003± 1.17×10^{-3}	0.0136± 3×10^{-4}	0.0082± 1.73×10^{-4}
Intercept± SD (S_a)	-0.0041± 1×10^{-4}	0.0053± 2.52×10^{-4}	-0.0022± 3.79×10^{-4}	0.0032± 2.65×10^{-4}
SD of residuals ($S_{y/x}$)	4.57×10^{-3}	4.42×10^{-3}	3.31×10^{-3}	6.50×10^{-3}
Correlation coefficient (r^2)	0.9997	0.9995	0.9999	0.999
Accuracy (R% ± SD)	100.22±1.92	100.74±1.37	99.28±0.95	100.19±1.69
Precision (RSD %)*				
Intraday	0.46-0.97	0.26-1.43	0.49-1.45	0.49-1.56
Interday	0.66-1.02	0.51-1.84	0.74-1.83	0.36-1.95

**average of 12 determination*

Table 2- Assay parameters of the proposed spectrophotometric methods for the determination of menbutone.

Parameter	BF Method	MB Method	SFNO Method	KIO_3/KI Method
λ_{max} (nm)	557	650	516	350
Linearity range (µg mL^{-1})	8-40	01-8	8-40	3-20
A (1%, 1cm)	265	1777	289	548
Regression parameters Slope ± SD (S_b)	0.0267± 3.22×10^{-4}	0.1768± 2×10^{-4}	0.0287± 3.61×10^{-4}	0.0542± 1.24×10^{-4}
Intercept± SD (S_a)	0.0001± 5×10^{-4}	0.0035± 2.08×10^{-4}	0.0106± 3.22×10^{-4}	0.0061± 5.13×10^{-5}
SD of residuals ($S_{y/x-}$)	1.82×10^{-3}	5.41×10^{-3}	4.25×10^{-3}	5.25×10^{-3}
Correlation coefficient (r^2)	0.9999	0.9998	0.9994	0.9997
Accuracy (R% ± SD)	100.08±0.75	100.03±0.89	100.89±1.37	99.96±0.41
Precision (RSD %)				
Intraday	0.29-1.97	0.40-1.21	0.25-1.63	0.66-1.54
Interday	0.53-1.43	0.70-1.58	0.50-2.04	0.12-1.25

**average of 12 determination*

Table 3- Statistical analysis of results obtained by the proposed charge transfer, NQS and manufacturer [21] methods for the determination of flunixin-M in its pharmaceutical preparation

Parameters	DDQ	CA	CR	NQS	Manufacturer method [21]
Linearity range (µg Ml^{-1})	10-100	50-300	10-80	20-120	5-30
N	6	6	5	6	5
Mean %	99.14	100.22	99.64	100.99	99.73
SD	1.64	1.23	0.71	1.07	1.18
Variance	2.68	1.51	0.5	1.15	1.39
t-	0.66	0.68	0.21	1.86	-
F-	1.93	1.01	2.78	1.21	-
Standard addition Mean ± SD	99.57± 0.83	96.22 ± 0.95	99.60 ± 1.06	104.12 ± 0.80	

The theoretical t- and F- values at P=0.05 were 2.262 and 6.16, respectively for DDQ, CA and NQS methods, while t- and F- values for CR method were 2.31 and 6.39, respectively; Delta Pharma [21] involved measuring UV-absorbance of the drug at 289 nm in ethanol.

Table 4- Statistical analysis of results obtained by the proposed and manufacturer method [12] for the determination of menbutone in its pharmaceutical preparations

Parameters	BF Method	MB Method	SFNO Method	KIO_3/KI Method	Manufacturer method [12]
Linearity range (µg mL^{-1})	08-40	01-08	08-40	03-20	2.5-25
N	5	5	5	6	6
Mean %	98.98	99.7	100.42	100.2	100.04
SD	1.1	1.24	2.01	1.51	1.37
Variance	1.21	1.55	4.03	2.29	1.88
t-	1.4	0.44	0.37	0.19	-
F-	1.55	1.22	2.14	1.22	-
Standard addition Mean ± SD	101.29 ± 1.22	100.67 ± 0.87	102.76 ± 2.04	97.90 ± 0.43	

The theoretical t- and F- values at P = 0.05 were 2.26 and, 5.19, respectively for BF, MB and SFNO methods, while 2.23 and 6.39 for KIO_3/KI method; Menbutone was determined by measuring the UV-absorbance of its aqueous solution at 321 nm.

Conclusion

The suggested methods have the advantage of being simple, accurate, sensitive and suitable for routine analysis in quality control laboratories. These methods utilize a single step reaction that can be used as general methods for the spectrophotometric, determination of flunixin-M or menbutone in bulk powder and in pharmaceutical formulations

Conflict of Interest : None Declared

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